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Hydrocarbon profiles and phylogenetic analyses of diversified cyanobacterial species

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HIGHLIGHTS

- The hydrocarbon profiles of 19 cyanobacterial species were studied.
- The conservation of the sequences of 16S rDNA and the two genes encoding the alkane-synthesizing enzymes was analyzed.
- The hydrocarbon distribution pattern in cyanobacteria was proposed with an evolutionary perspective.

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ABSTRACT

The combination of environmental concerns and the growing demand for energy make the development of biofuels, an attractive alternative to fossil fuels, a goal for many researchers. The direct photosynthetic production of hydrocarbons, which are the major components of fossil fuels, is considered to be a promising and innovative strategy for the development of biofuels with advanced fuel properties and solar-driven energy input. Cyanobacteria have existed continuously since the early evolution of the biosphere and the biosynthetic pathways of hydrocarbons in these prokaryotes have been genetically and biochemically identified. In this study, the hydrocarbon compositions of 19 freshwater cyanobacterial species distributed among 13 genera were analyzed. Based on their hydrocarbon profiles, these cyanobacterial species were classified into 5 major subgroups. Combined with the previously reported hydrocarbon compositions in different cyanobacterial species, we found that branched-chain alkanes were limited predominantly in filamentous species but rarely in unicellular species. Phylogenetic analysis using traditional small-subunit ribosomal RNA (16S rDNA) of these strains presented clustering similar to their hydrocarbon production profiles. Acyl–acyl carrier protein reductase (AAR) and aldehyde deformylating oxygenase (ADO) are two key enzymes involved in the biosynthesis of hydrocarbons in cyanobacteria. A comparison of phylogenies revealed that the topology of 16S rDNA showed a general congruence with that of AAR but not with that of ADO. The results not only provide an evolutionary perspective with which to study the physiological function of cellular hydrocarbons but also display the engineering capacity to molecularly design diversified hydrocarbon fuel products in cyanobacteria.

1. Introduction

Together, rapidly growing energy demands and environmental concerns caused by the use of fossil fuels render the development of renewable and sustainable biofuels, particularly advanced biofuels with fuel properties similar to fossil fuels, increasingly attractive and urgent. Hydrocarbons produced from biomass resources or directly from solar energy and carbon dioxide through photosynthetic biological systems are becoming a significant goal of research and development in both academia and industry. Cyanobacteria, which are capable of the photosynthetic production of hydrocarbons and exhibit multiple adaptive morphological, biochemical and metabolic properties, have garnered particular attention because of their huge potential for renewable energies [1–3].

Cyanobacteria are common inhabitants of pristine terrestrial and aquatic environments on a global scale and include unicellular and colonial species, which form filaments, sheets or even hollow balls in natural environments [4]. They are known to be monophyletic but morphologically diverse, and traditionally, they are divided into five major subsections according to their

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morphological distinctions [5,6]. Cyanobacterial strains of subsection I (e.g., *Chroococcales*, *Prochlorophytes* and *Gloeobacterales*) and II (e.g., *Pleurocapsales*) are unicellular. Subsections III, IV and V form filaments of various morphological complexities. It is reported that the strains of subsection III (e.g., *Oscillatoriales*) have only vegetative cells, but in subsections IV (e.g., *Nostocales*) and V (e.g., *Stigonematales*), vegetative cells can differentiate into morphologically distinct heterocysts [7]. To date, at least 126 cyanobacterial genomes have been sequenced which cover all five traditional subsections, the unicellular, baeocystous, filamentous, heterocystous and ramified phenotypes [6].

Early research in the 1960s showed that hydrocarbons could be produced in a variety of cyanobacterial strains (e.g., *Nostoc* sp., *Anacystis* sp., *Anacystis nidulans*, *Trichodesmium erythaeum*, *Microcoleus chthonoplastes*, *Plectonema terebrans*, *Oscillatoria williamsii*, *Lyngbya lagerhaimii*, etc.). This research also revealed that cyanobacteria mainly produced hydrocarbons with carbon chain lengths varying from C15 to C18 with a predominance of n-C17 compounds [8–10]. Two marine unicellular strains, *Agmenellum quadruplicatum* (currently named *Synechococcus* sp. PCC 7002) and *Coccochloris elabens* (currently named *Synechococcus* sp. PCC 7003), are known to synthesize C19 hydrocarbons with either one or two double bonds [8]. These initial investigations concerning hydrocarbon production in cyanobacteria were focused on their potential geochemical and taxonomic significance [11,12], and there were few further studies on hydrocarbon biosynthetic processes at the molecular level in the following decades.

Eukaryotic microalgae are an important resource for biofuel production [13–15]. Unlike microalgae, which accumulate large amounts of triacylglycerols (TAGs) through lipid metabolism involving several different cellular compartments, cyanobacteria contain few or no TAGs. Fatty acid metabolism in cyanobacteria is associated with a type II fatty acid synthesis (FASII) and is conducted by a multienzyme system in the cytoplasm. Fatty acid molecules must be activated to fatty acyl-thioesters by fatty acyl-CoA synthetase (ACS) or fatty acyl-ACP synthetase (AAS) before hydrocarbons can be synthesized. Free fatty acids exist mainly in the form of C18 with a small amount of C16 in cyanobacteria, thus the predominant hydrocarbon is C17. One precursor of hydrocarbon biosynthesis in cyanobacteria is thought to be fatty acyl–acyl carrier proteins (ACPs), which are essential metabolites for the production of lipid-based biofuels [3,16–18].

Until recent years, the common pathway for hydrocarbon production in cyanobacterial species was only preliminarily identified. This pathway consists of an acyl–acyl carrier protein (ACP) reductase (AAR), which reduces acyl-ACPs to aldehydes [1], and an aldehyde deformylating oxygenase (ADO), which converts aldehydes into alka(e)nes [1,19–23]. To our knowledge, there are four other hydrocarbon biosynthetic pathways in prokaryotes: (i) Isoprenoid biosynthesis involves the condensation of isoprene units to generate hydrocarbons with multiples of five carbon atom units (C10, C15, C20, etc.) [24]. (ii) In representative bacteria from diverse phyla (*Verucomicrobia*, *Planctomyces*, *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, etc.), different types of long-chain olefinic hydrocarbons are synthesized from the head-to-head condensation of fatty acids, which depends on Ole proteins [25,26]. (iii) In *Jeotgalicoccus* sp. ATCC 8456, terminal olefins are synthesized through a direct fatty acid decarboxylation mechanism catalyzed by P450 fatty acid decarboxylase [27]. (iv) In the single-celled cyanobacterial strain *Synechococcus* sp. PCC 7002 mentioned above, hydrocarbon biosynthesis employs an elongation–decarboxylation mechanism, which is involved in the conversion of fatty acyl-ACPs to olefins [28]. The recent identification of different hydrocarbon biosynthetic pathways in diverse bacteria and cyanobacteria display the potential and feasibility to improve hydrocarbon production in cyanobacteria by employing genetic, enzymatic and metabolic engineering strategies. Our previous work demonstrated the viability of a cyanobacteria-based platform that targets fatty acid–based biofuels and discussed the possibility of controlling and regulating the properties of hydrocarbon fuels by choosing different starting cyanobacterial strains or by manipulating hydrocarbon biosynthesis pathways [2,29]. However, why cyanobacterial species produce hydrocarbons and how different types of hydrocarbons are synthesized are phenomena that have not yet been elucidated.

In this study, the hydrocarbon composition of 19 freshwater cyanobacterial species distributed among 13 genera was surveyed. By structurally characterizing the species of hydrocarbons from each strain and subsequently comparing the strains and their composition to previously reported work, we found that most fatty alka(e)nes with branched chains appear to exist predominantly in filamentous strains but rarely in unicellular strains. Additionally, we examined the evolutionary relationship of AAR and ADO with hydrocarbon biosynthesis in cyanobacteria, which might provide an evolutionary perspective on the hydrocarbon biosynthetic pathways in cyanobacteria.

2. Materials and methods

2.1. Chemicals and reagents

Eicosane was obtained from Sigma–Aldrich (USA). All other chemicals were obtained from either Merck (Germany) or Amaresco (USA). Oligonucleotide synthesis and sequencing were performed by Sunny (Shanghai, China). *Taq* and *Pfu* DNA polymerases were purchased from Fermentas (Canada). The kits used for molecular cloning were from Omega or Takara (Japan).

2.2. Strains, culture conditions and growth curves

Synechocystis sp. PCC 6803, *Anabaena* sp. PCC 7120 and *Synechococcus elongatus* PCC 7942 were generous gifts from Professor Xudong Xu of the Institute of Hydrobiology, Chinese Academy of Sciences. The *Nostoc punctiforme* strain ATCC 29133 was kindly provided by Professor John C. Meeks of the University of California, Davis. All other cyanobacterial strains used in this study were purchased from the Freshwater Algal Culture Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences.

The original source of all the strains used in this study is FACHB monocultures. Three methods were used to ensure that there was no contamination. First, each strain was regularly inspected via microscopy for the entire duration of the experiments to confirm that they were not contaminated by other algae. Second, during the 16S rDNA analysis, three replicates of sequenced 16S rDNA fragments were consistent with one another for each strain; this showed that there was no contamination at the molecular level. Third, we used Luria–Bertani medium to check for contamination by heterotrophic bacteria and found that each strain was axenic.

The cyanobacterial strains used in this study were grown at 30 °C in 500 mL flasks, each of which contained 300 mL of air-infused BG11 medium [30] under 30–50 $\mu E/m^2/s$ of white light. The dry cell weights (DCWs) of cyanobacterial cells were used for the determination of cell growth curves. The seed cultures were grown under the conditions mentioned above for approximately 5 days using 5% (v/v) inoculums. Every 2 days, 5 mL of the thoroughly mixed culture was filtered onto dried and pre-weighed nitrocellulose filter membranes (25 mm in diameter, 0.22 μm in mesh) and then dried at 110 °C for approximately 24 h to constant weight; the DCW was calculated by subtracting the initial weight of the filter. The DCW data shown in Table 2 represents the means ± standard deviations of the values from three replicates.